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Beverly Weinberger
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January 6, 2005

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Re:	Appellants:	Hong Xue, Hui Kwok Min, Hongyan Wang and Hui Zheng	
	Application No.:	09/909,862	Filed: July 20, 2001
	Confirmation No.:	8767	
	Title:	COMPOUND FOR TREATMENT OF ANXIETY AND METHODS OF PREPARATION AND USE THEREOF	
	Docket No.:	3053.1000-001	

Sir:

Transmitted herewith is an Amended Appeal Brief for filing in the subject application. An Appeal Brief was mailed to the United States Patent and Trademark Office on 1 December 2004, pursuant to the Notice of Appeal received by the U.S. Patent and Trademark Office on 1 July 2004.

The Appeal Brief mailed to the Patent Office on 1 December 2004 was believed to be in compliance with 37 C.F.R. § 41.37(c). However, since 1 December 2004, it has come to the attention of Appellants' Attorney that Naturon Limited, not PharmacoGenetics Limited, is the real party in interest. This Amended Appeal Brief is submitted to the U.S. Patent and Trademark Office to correct this unintentional error.

Please charge any deficiency or credit any overpayment in the fees that may be due in this matter to Deposit Account No. 08-0380. A copy of this letter is enclosed for accounting purposes.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By Carol A. Egner

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Concord, MA 01742-9133
Dated: January 6, 2005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellants:	Hong Xue, Hui Kwok Min, Hongyan Wang and Hui Zheng		
Application No.:	09/909,862	Group:	1617
Filed:	July 20, 2001	Examiner:	Shengjun Wang
Confirmation No.:	8767		
For:	COMPOUND FOR TREATMENT OF ANXIETY AND METHODS OF PREPARATION AND USE THEREOF		

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on January 6, 2005 Beverly Weinberger
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AMENDED APPEAL BRIEF

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

An Appeal Brief was mailed to the U.S. Patent and Trademark Office on 1 December 2004, pursuant to the Notice of Appeal received in the U.S. Patent and Trademark Office on 1 July 2004, and in support of the appeal from the final rejection set forth in the Office Action mailed on 30 March 2004. The fee for filing a brief in support of an appeal was then enclosed. A Petition for Extension of Time and the appropriate fee were also filed with the Appeal Brief on 1 December 2004.

The Appeal Brief mailed to the Patent Office on 1 December 2004 was believed to be in compliance with 37 C.F.R. § 41.37(c). However, since 1 December 2004, it has come to the attention of Appellants' Attorney that Naturon Limited, not PharmacoGenetics Limited, is the real party in interest. This Amended Appeal Brief is submitted to the U.S. Patent and Trademark Office to correct this unintentional error. No other changes have been made to the Appeal Brief as it was originally mailed to the U.S. Patent and Trademark Office on 1 December 2004.

I. REAL PARTY IN INTEREST

The real party in interest is Naturon Limited. Naturon Limited is the Assignee of the entire right, title and interest in the subject application.

II. RELATED APPEALS AND INTERFERENCES

Appellants, the undersigned Attorney and Assignee are not aware of any related appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 13-16 and 19-22 have been finally rejected, and a copy of these claims appears in the Appendix of this Brief. Claims 13-16 were amended in the Amendment filed on 17 January 2003. Claims 20-22 were added in the Amendment filed on 12 June 2003. Claim 19 appears as originally filed. Claims 1-12, 17 and 18 were canceled.

IV. STATUS OF AMENDMENTS

No Amendment After Final has been filed. A Reply After Final Rejection Under 37 C.F.R. § 1.116, not requesting any amendments, was filed on 28 May 2004. An Advisory Action was mailed from the United States Patent and Trademark Office on 9 June 2004, stating that the Reply failed to place the application in condition for allowance. A Reply to Advisory Action not requesting any amendments was filed on 2 August 2004. A second Advisory Action was mailed from the United States Patent and Trademark Office on 15 September 2004, stating that the Reply failed to place the application in condition for allowance.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The invention is a method to treat anxiety in a human by administration to the human of a flavonoid compound, 5, 7-dihydroxy-8-methoxyflavone, commonly referred to as wogonin. The claims include variations on this method in the amount and method of administering the wogonin.

Support for Claims 13-16 and 19-22 can be found on page 5, line 8 to page 6, line 2, and in the experimental evidence on page 17, line 17 to page 19, line 11. See also page 8, lines 10-13 for Claims 14-16 and Claims 20-22.

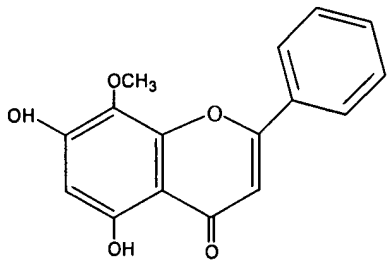
VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

There is one basis of rejection raised by the Examiner during prosecution is to be reviewed on appeal. The issue is whether Claims 13-16 and 19-22 are obvious under 35 U.S.C. § 103(a) in view of Cassels *et al.* (US 5,756,538).

VII. ARGUMENT

Claims 13-16 and 19-22 have been rejected under 35 U.S.C 103(a), as it is said that they are unpatentable over Cassels *et al.* (US 5,756,538).

The subject application has claims drawn to a method of treating anxiety in a patient in need thereof, comprising administering an effective non-toxic dose to the patient of a compound commonly referred to as wogonin, having the formula:



In the cited patent (US 5,756,538) Cassels *et al.* describe flavonoids, flavonone derivatives and biflavonoids. The Cassels *et al.* patent describes in particular seven preferred subgenuses of compounds having general formula (I) (column 3, lines 16-29) and a number of preferred compounds (column 3, lines 31-34). One of the seven subgenuses set forth as being preferred is a subgenus "wherein R^3 and R^1 are both hydroxy." R^3 and R^1 are positions 7 and 5, respectively, of flavone. A general statement is made in the patent (column 2, lines 1-9) that the described compounds (presumably referring to *all* of the compounds described in the patent) have anxiolytic properties. However, only diazepam, chrysin, 2'-fluorochrysin, 2'-bromochrysin, 6, 8-dibromochrysin, apigenin, and 7-bromoflavone were tested for anxiolytic effects in rats.

The Examiner stated in the Office Action of 22 August 2003:

> However, it would have been prima facie obvious to a person of ordinary skill in the art, at the time the claimed invention was made, to treat anxiety by employing a flavone with hydroxyl groups at 5 and 7, and a methoxyl group at position 8 (R4 as depicted by Cassels)." A person of ordinary skill in the art would have been motivated to treat anxiety by employing a flavone with hydroxyl groups at 5 and 7, and a methoxyl group at position 8 (R4 as depicted by Cassels) because Cassels expressly prefer flavone with 5 and 7 dihydroxyl groups and methoxyl group is known to be useful as a substituent at position 8 (R4). The selection of methoxyl group herein is seen to be a selection from amongst equally suitable functional groups and as such obvious. Ex parte Winters 11 USPQ 2nd 1387 (at 1388).

This is not the correct analysis to attempt to establish a prima facie case of obviousness for a method of treating anxiety in a patient. *The Manual of Patent Examining Procedure* (MPEP) devotes section 2144.08 to "Obviousness of Species When Prior Art Teaches Genus." Patent examiners are to follow a flowchart (page 2100-150 of MPEP -- February 2003 revision of 8th edition) to determine whether a prima facie case of obviousness can be found. The flowchart states at the top, "If the closest prior art is a single reference disclosing a genus, determine whether the claimed species or subgenus would have been obvious to one of ordinary skill in the pertinent art at the time the invention was made by performing the following analysis." In the present case, the cited prior art is a single reference disclosing a genus of methods; a species of method within this genus is claimed.

The patent examiner is to first consider the "Graham factors." A claimed invention is unpatentable if the differences between it and the prior art "are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art." See *Graham v. John Deere Co.*, 383 U.S. 1, 14, 86 S.Ct 684, 15 L.Ed.2d 545, 148 USPQ 459, 465 (1966), which set forth factors to be considered: (1) the scope and content of the prior art; (2) the level of ordinary skill in the prior art; (3) the differences between the claimed invention and the prior art; and (4) objective evidence of nonobviousness. The Examiner has not expressed reliance on any of these "Graham factors" to make a prima facie case of obviousness.

To establish a prima facie case of obviousness, the patent examiner is next to determine whether there would have been motivation to select the claimed species.

If Cassels *et al.* teach a method of treating anxiety in a patient by administering to the patient a compound that would fall into the described (column 3, lines 16-19) "compounds of general formula (I) wherein R^1, R^2, R^3, R^4 and R^5 may independently be H, OH or halo (including F, Cl, Br or I)," then a large number of methods are taught, corresponding to the large number of compounds that would fall into this genus of compounds. The Examiner has not pointed out any express teachings in the prior art that would have motivated the selection of the subgenus method of treating anxiety by administering "flavone with 5 and 7 dihydroxyl groups" from among the *seven* subgenus methods indicated as preferred, and further, has not pointed out any express teachings that would have motivated the selection of a method of treating anxiety by administering wogonin from the many methods to be selected from, based on the many permutations of the compound of general formula (I).

Further, it is incorrect to assume that "[t]he selection of methoxyl group herein is seen to be a selection from amongst equally suitable functional groups . . . ." It is the obviousness or nonobviousness of the invention *as a whole*, not the selection of a methoxyl group, that must be considered. Here, the invention is a method of treating anxiety in a patient; the invention is not a compound or subgenus of compounds. Cassels *et al.* do not report data that would lead to the conclusion that all compounds within the subgenus of flavones with hydroxyl groups at positions 5 and 7 are anxiolytic agents. Cassels *et al.* do not report any data on the biological activity of wogonin that would indicate its efficacy as an anxiolytic agent. Table II in column 10 of Cassels *et al.* shows the results of an experiment in which various flavonoids were tested for their ability to compete with ^{3}H-flunitrazepam for the benzodiazepine receptor. The IC_{50} values indicating affinity for the benzodiazepine receptor, from which anxiolytic activity may be inferred, vary by 8,000 fold for the fifteen compounds tested, demonstrating the unpredictable effects of substituting one functional group for another. Thus, the activity of wogonin as an anxiolytic agent could not have been predicted.

A prima facie case of obviousness has not been established for a method of treating anxiety in a patient. Cassels *et al.* recite a great number of *compounds* as being preferred (column 3, lines 16-34). No *methods of treating anxiety* are recited as being preferred, and there is no instruction on how to choose a method to treat anxiety. Therefore, there is no suggestion in

Cassels *et al.* that the method of Applicants or a method using a structurally similar compound would be successful.

Cassels *et al.* do not present a random sample of flavonoids with randomly selected substituents for positions R^1 - R^8, along with their physiological properties. Rather, out of a great number of compounds in this structural family, they show *in vivo* data for only two selected compounds, chrysin and apigenin. One of ordinary skill in the art could not extrapolate from these very limited data what structural features are necessary in a flavonoid to produce an anxiolytic effect.

The Examiner states:

> A person of ordinary skill in the art would have been motivated to treat anxiety by employing a flavone with hydroxyl groups at 5, and 7, and a methoxyl group at 8 position (R4 as depicted by Cassels) because Cassels expressly prefer flavone with 5 and 7 dihydroxyl groups and methoxyl group is known to be useful as a substituent at 8 position (R4) (*object evidence of obviousness*). The selection of methoxyl group herein is seen to be a selection from amongst equally suitable functional groups and as such obvious. *Ex parte Winters* 11 USPQ 2nd 1387 (at 1388).

Ex parte Winters is a case directed to composition of matter claims in which the compositions were organic compounds. The claims of the instant patent application are to *methods of treating anxiety*. The Examiner has no basis for the conclusion that a compound with a methoxyl group at position 8 (R4 in Cassels, *et al.*) would be effective in a method to treat anxiety. The physiological properties of compounds with hydroxyl groups at the 5 and 7 positions could not have been predicted by one of ordinary skill in the art. For example, data are presented in column 10 of Cassels *et al.* for the displacement of flunitrazepam from synaptosomal membrane fractions by the compounds apigenin, 6, 8-dibromochrysin, isoquercitrin, rutin, 2'-fluorochrysin and 2'-chlorochrysin. The IC_{50} values of these compounds--all having hydroxyl groups at the 5 and 7 positions--vary over a 100-fold range. These results could not have been predicted on the basis of structure.

Contrary to what the Examiner has stated, it could not have been predicted at the time of the invention, and it is not true, that the entire class of flavonoids with hydroxyl groups at the 5 and 7 positions have anxiolytic activity. Consider 5,7-dihydroxy-6-methoxyflavone (oroxylin

A), described in Huen *et al.* (Huen, M.S.Y. *et al., Biochem. Pharmacol. 66(1)*:125-132, 2003; see attached Exhibit A, previously presented with the Reply After Final Under 37 C.F.R. § 1.116 mailed to the Patent Office on 28 May 2004). The only structural difference between wogonin of the claims and oroxylin A is the position of the methoxyl group (at the 8 position in wogonin, and at the 6 position in oroxylin A). Like wogonin, oroxylin A is extracted from *Scutellaria baicalensis.* However, oroxylin A acts as an antagonist of agents that bind at the $GABA_A$ receptor benzodiazepine binding site as agonists. Oroxylin A has no stimulant, depressive, sedative, anxiolytic or myorelaxant effects of its own. Nor does it affect motor coordination of picrotoxin-induced seizures. Oroxylin A abolishes the anxiolytic and motor uncoordination effects of diazepam, and partially counteracts the myorelaxation effect of diazepam.

Using the Examiner's reasoning, it might have been predicted that oroxylin A would be effective in treating anxiety. However, this is not the case, as shown by Exhibit A. This provides evidence that compounds in the class are not functional equivalents and therefore, one skilled in the art cannot draw generalized conclusions on the functionality of Appellants' compounds. Therefore, the method of Claims 13-16 and 19-22 is not obvious.

The Examiner seems to be saying that to carry out a method of treating anxiety, one of ordinary skill in the art would be able to consider the class of compounds described in Cassels *et al.* and choose among a group of compounds with similar structures. The Examiner is making the assumption that compounds of similar structures have similar anxiolytic effects.

This assumption is incorrect. Of the great many possible flavonoids described by structure in Cassels *et al.*, only a few were reported to have any physiological effect. No negative results were reported, leaving one to guess at how many were tested and not found to be useful for anti-anxiety effects. Only six flavonoids were shown to have anxiolytic activity in mice. The compounds were chrysin, apigenin, 2'-chlorochrysin, 2'-fluorochrysin, 6, 8-dibromochrysin and 7-bromoflavone. The only common structural feature shared by the six flavonoids was the underlying structure of flavone. Thus, it is inherent in the art that the structure of flavonoids is not correlated with their anxiolytic function.

This inherent lack of correlation between structure and physiological function among the flavonoids is further demonstrated by the studies on oroxylin A by Huen *et al.* As described

above, wogonin and oroxylin A produce completely opposite physiological effects, despite their structural similarity.

The only reason why, as the Examiner suggests, "A person of ordinary skill in the art would have been motivated to treat anxiety by employing a flavone with hydroxyl groups at 5 and 7, and a methoxyl group at position 8 (R4 as depicted by Cassels) . . ." is if there had been reason to assume that a compound of such a structure would have anxiolytic effects. Cassels *et al.* gave one of ordinary skill in the art no reason to make such an assumption. There is no evidence in Cassels *et al.* that a flavonoid with substituents at the 5, 7 and 8 positions would have an anxiolytic effect, or that a methoxyl group at position 8 or any other position would produce an anxiolytic flavonoid.

Respectfully submitted,

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By Carol A. Egner

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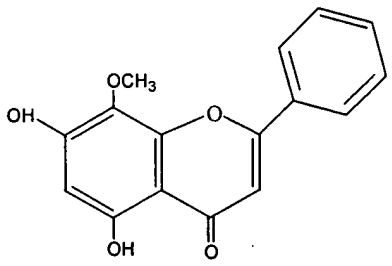
Concord, MA 01742-9133

Date: January 6, 2005

CLAIMS APPENDIX

13. (Previously presented) A method of treating anxiety in a patient in need thereof comprising administering an effective non-toxic dose to the patient of a compound of the formula:



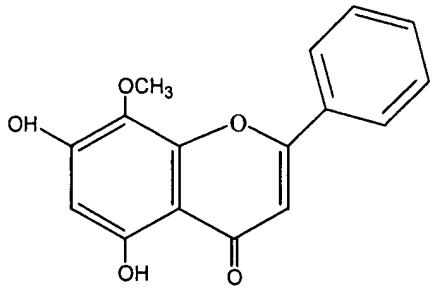
14. (Previously presented) The method of Claim 13, wherein the dose administered to the patient is from about 0.15 mg/kg to about 1.0 mg/kg.

15. (Previously presented) The method of Claim 13, wherein the dose is administered in a single aliquot.

16. (Previously presented) The method of Claim 13, wherein the dose is administered in two or more aliquots.

19. (Original) A method of treating anxiety in a patient comprising administering an effective non-toxic dose of wogonin to the patient.

20. (Previously presented) A method of treating anxiety in a patient in need thereof comprising administering to the patient from about 0.1 mg/kg to about 10.35 mg/kg of a compound of the formula:



21. (Previously presented) The method of Claim 20, wherein the compound administered to the patient is from about .8 mg/kg to about 3.3 mg/kg.

22. (Previously presented) A method of treating anxiety in a patient comprising administering a dose from about 0.1 mg/kg to about 10.35 mg/kg of wogonin to the patient.

EVIDENCE APPENDIX

Exhibit A: (Huen, M.S.Y. *et al.*, *Biochem. Pharmacol. 66*(1): 125-132, 2003)

Exhibit A was provided for the Examiner with a Reply After Final Rejection Under 37 C.F.R. § 1.116, mailed to the U.S. Patent and Trademark Office on May 28, 2004.

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5,7-Dihydroxy-6-methoxyflavone, a benzodiazepine site ligand isolated from *Scutellaria baicalensis* Georgi, with selective antagonistic properties

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Abstract

As part of an effort to identify naturally occurring $GABA_A$ receptor benzodiazepine binding site (BDS) ligands from traditional medicinal herbs, we previously reported that flavonoid derivatives isolated from *Scutellaria baicalensis* (*S. baicalensis*) Georgi exhibited significant affinities for the BDS. The present study describes the characterization of 5,7-dihydroxy-6-methoxyflavone (oroxylin A), one of the major components of the herbal extract. Oroxylin A inhibited [^{3}H]flunitrazepam binding to rat cerebral cortical membrane with a IC_{50} value of 1.09 ± 0.07 μM. A GABA ratio of 1.09 ± 0.04 suggests that oroxylin A interacts as an antagonist at the recognition site. In neuropharmacological studies, oral administration of oroxylin A (3.75–60 mg kg^{-1}) did not result in significant changes in animal models routinely employed for benzodiazepine (BD) evaluation. However, oroxylin A selectively abolished the anxiolytic, myorelaxant and motor incoordination, but not the sedative and anticonvulsant effects elicited by diazepam, a BDS agonist. These results add oroxylin A to the list of CNS active flavonoids, and as the first naturally occurring member endowed with selective antagonistic actions *via* the BDS.

Keywords: Flavonoids; Benzodiazepine binding site; Anxiolytics; Pharmacology; Naturally-occurring; *Scutellaria baicalensis* Georgi

1. Introduction

BDs have widely been used as potent anxiolytics, sedative, hypnotics and anticonvulsants. Their recognition site, BDS, is one of the multiple binding sites on the $GABA_A$ receptor, and serves as a regulatory element for the inhibitory $GABA_A$ receptor function.

Despite the usefulness of conventional BD as potent anxiolytics, the accompanying compromising effects, including sedation, myorelaxation and memory loss following chronic administration, has initiated a search for alternatives devoid of these untoward actions. Since the discovery of amentoflavone, which manifested high-affinity for the BDS [1], much research has been devoted to the quest for alternative BDS ligands from natural sources. Chrysin was amongst the first member of the class of compound to demonstrate CNS activities *via* the BDS [2]. To date, a list of both natural and synthetic flavonoids have been shown to exert BDS-mediated pharmacological actions with efficacies resembling to that of full agonists [3], partial agonists [4], and antagonists [5,6].

These ligands modulate in both directions and in varying amplitudes the binding of GABA to its receptor. It was observed that the presence of GABA enhanced the binding of a BDS agonist, reduced the binding of an inverse agonist, and exerted negligible effect on the binding of a BDS antagonist. The GABA shift experiment based on these allosteric modulatory properties provide a useful assay for correlating the *in vitro* behavior of a BDS ligand with its potential pharmacological profile [7].

Antagonists at the BDS are useful in the treatment of BD overdose and hepatic encephalopathy [8–10]. Ro15-1788, one of the most investigated synthetic antagonists, is the only prescribed drug amongst this class of compounds. However, due to the anxiogenic effects accompanying its administration, the development of novel antagonists as

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Abbreviations: GABA, γ-aminobutyric acid; BDS, benzodiazepine binding site; BD, benzodiazepine; Oroxylin A, 5,7-dihydroxy-6-methoxyflavone.

0006-2952/03/$ – see front matter
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new pharmacological agents for research and therapeutic applications is much needed.

The root of *S. baicalensis* Georgi is widely employed in traditional Chinese prescriptions. Flavonoids from this medicinal herb have been shown to possess a broad spectrum of antiviral, antioxidant, anti-inflammatory, and antiallergic actions [11–14]. As part of the screening of traditional herbal extracts for BDS activity, we previously reported that several flavonoids isolated from this herb exhibited moderate affinities for the receptor [15]. Behavioral studies demonstrated that wogonin, one of these flavonoids, exerted potent anxiolysis in mice without sedative and myorelaxant actions [16].

The present study describes the characterization of another orally active flavonoid derivative, oroxylin A, isolated from *S. baicalensis* Georgi, with selective antagonistic properties at the BDS.

2. Materials and methods

2.1. Drugs

Radioactive ligands [^{3}H]flunitrazepam (*N*-methyl-[^{3}H], 88.0 Ci mmol) and [^{3}H]Ro15-1788 (*N*-methyl-[^{3}H], 78.6 Ci mmol^{-1}) were obtained from NEN Life Science Products. Diazepam, GABA, Ro15-1788 and picrotoxin were from Sigma. Valium (diazepam) ampoules were from Hoffmann-La Roche. All organic solvents were of analytical grade.

2.2. Isolation and identification of oroxylin A

The roots of *S. baicalensis* Georgi were purchased from traditional Chinese medicine suppliers in Hong Kong as a dried crude herb and was authenticated by Prof. Y.Z. Guo, Faculty of Traditional Chinese Medicine, Shenyang Pharmaceutical University, PR China. A voucher specimen (SB1) is deposited in the Department of Biochemistry, The Hong Kong University of Science and Technology.

Two hundred grams of the herb were extracted three times consecutively each with 4 L of dichloromethane. The resulting extract was dried (6 g) and was subjected to polyamide gel column chromatography. The sample was eluted from the column using successive mixtures of petroleum ether and chloroform in increasing polarity, up to a ratio of 7:1, to give oroxylin A (10 mg; Fig. 1). Identification of oroxylin A was carried out by ESI-MS, ^{1}H and ^{13}C NMR spectral analysis and specroscopical data corresponds to that of previously described [17]. Purity of samples was >95%.

2.3. Synaptosomal membranes

Synaptosomal membranes were prepared as described previously [18], from male Sprague–Dawley rats. Briefly, the rats were decapitated and cerebral cortex dissected.

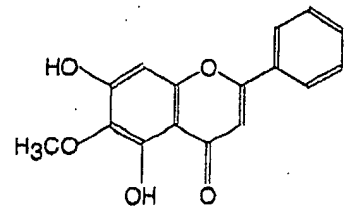

Fig. 1. The chemical structure of oroxylin A. 5,7-Dihydroxy-6-methoxy-flavone (MW 284).

Tissues were stored frozen until used. The tissues were thawed and homogenized in 0.32 M sucrose (20 mL g^{-1} of tissue) and centrifuged at 1000 *g* for 10 min at 4°. The supernatant was centrifuged at 140,000 *g* for 20 min at 4°. For [^{3}H]flunitrazepam binding studies, this 140,000 *g* pellet was resuspended in 2 vol. of 0.05 M Tris–Cl, pH 7.4 and stored frozen in aliquots of 100 μL until use. For [^{3}H]Ro15-1788 binding studies, the pellet was washed by resuspending in ice-cold distilled-deionized water and centrifuged again at 140,000 *g* for 20 min at 4°. Washing was repeated two more times in order to remove endogenous GABA from the synaptosomal membrane preparation. After the final centrifugation step, the pellets were resuspended in 0.05 M Tris–Cl (pH 7.4) and stored frozen (−80°) in 1 mL aliquots. On the day of the assay, they were thawed, washed one more time in distilled-deionized water as above and then resuspended for use in assays.

2.4. Radioreceptor binding studies

2.4.1. [^{3}H]Flunitrazepam binding assay

[^{3}H]Flunitrazepam binding was measured using filtration methods as described previously [18]. Briefly, aliquots (0.7–0.9 mg mL^{-1}) of synaptosomal membranes were resuspended in 0.05 M Tris–Cl, pH 7.4 and incubated with 1 nM [^{3}H]flunitrazepam at 4° for 60 min in the absence and presence of test drug. Nonspecific binding was measured in the presence of 10 μM diazepam, amounted to <10% of total binding. After incubation, the mixtures were filtered onto Whatman GF/B filters using a Brandel 24-well harvester and rapidly washed with ice-cold 0.05 M Tris–Cl, pH 7.4 buffer for 5 s. Individual filters were incubated overnight with 5 mL scintillation cocktail before measurement of radioactivity in a Wallac Reckbeta 1209 liquid scintillation counter. For saturation analysis, tissue aliquots were incubated with or without test drug and increasing concentrations of [^{3}H]flunitrazepam (0.1–10 nM). Results were determined by nonlinear regression analysis (sigmoidal curve fitting) of specifically bound radioligand, as % of control vs. log(*M*). K_d and B_{max} from the saturation experiments were determined using Prism 3.0 (Graphpad Software).

2.4.2. [^{3}H]Ro15-1788 binding assay

The [^{3}H]Ro15-1788 binding assay was identical to that of [^{3}H]flunitrazepam binding except extensively washed

membranes were incubated in 1 nM [^{3}H]Ro15-1788. For GABA shift experiments, test drug was incubated in the presence or absence of 10 μM GABA. GABA ratio was determined by dividing the IC_{50} of test drug in the absence of GABA by the IC_{50} of the test drug in the presence of GABA.

2.5. Behavioral studies

2.5.1. Drug solutions

Orally administered drugs were dissolved into distilled-deionized water by ultrasonication with the addition of one drop of Tween 80 to give an injection volume of 10 mL kg^{-1}. Diazepam (i.p.) and picrotoxin (s.c.) were dissolved in 10% DMSO and physiological saline, respectively.

2.5.2. Animals

Male ICR mice (16–20 g; Animal Care Centre, HKUST) were housed in groups of four to five with food and water *ad lib.* and kept on a 08:00 to 20:00 hr light cycle. Experiments were conducted between 08:30 and 12:00 hr.

2.5.3. Locomotor activity test

To differentiate between potential stimulant or depressant drug effects, the locomotor activity test was performed. The model ZIL-2 apparatus (Beijing Institute of Materia Medica, China) was employed, with dimensions of 60 cm × 60 cm × 12 cm, consisting of four circular plastic boxes (diameter 25 cm) each with six evenly spaced infrared photocells. Locomotor activity was counted automatically during a 5-min test period in terms of the number of transitions across the light beams detected by the photocells.

2.5.4. Holeboard test

The mouse holeboard was a wooden box with a 60 cm × 60 cm square floor and 30 cm high walls. There were four equally spaced holes in the floor, each 3 cm in diameter. Each mouse was placed singly at the center of the board, facing away from the observer, and the number of head-dips and the number of rears made in a test period of 5 min were recorded. After each trial, the floor of the apparatus was wiped and dried to remove traces of the previous path. Due to wide variations in motor activity and head-dipping throughout the day [19], the mice were tested between 09:00 and 12:00 hr.

2.5.5. Elevated plus-maze

The maze had two opposite arms, 25 cm × 10 cm, crossed with two enclosed arms of the same dimensions but having 20-cm high walls. The arms were connected to a central platform, 5 cm × 5 cm, giving the apparatus the shape of a plus sign. The maze was kept in a dimly-lit room and elevated 40 cm above ground. Following the holeboard test, mice were placed individually at the center of the maze facing an enclosed arm. Number of entries and time spent in the open arms and closed arms were recorded in a 5-min period. An arm entry was defined by having all four paws inside the arm. At the end of the test, the number of entries and the time spent in the open arms were expressed as a percentage of the total number of entries in the arms and the total time spent in either arm, respectively. It is well established that the proportion of entries into the open arms in the elevated plus-maze is a good indicator of antianxiety action while the number of closed arm entries is related to drug effects on locomotor activities [20,21].

2.5.6. Horizontal wire test

Mice were lifted by the tail and allowed to grasp a horizontally strung wire (1 mm diameter, 15 cm long and placed 25 cm above floor) with their forepaws, and then released [22]. Each mouse was tested prior to drug administration. Normal animals would actively grasp the wire with their hind limbs. Only these mice successful in grasping the wire with their hind limbs were scored. A myorelaxant drug impairs the ability of the mice to grasp the wire, and muscle relaxation is commonly associated with sedation.

2.5.7. Rotarod test

The rotarod test employed a custom-built apparatus consisting of an elevated cylinder (diameter 2.5 cm) 0.5 m above ground, with a textured surface that rotated at 10 rpm. After administration of the test substance, each mouse was tested for its ability to stay on the rotarod for a duration of 1 min.

2.5.8. Picrotoxin (PTX)-induced seizure test

Seizures were induced by the s.c. injection of picrotoxin (6 mg kg^{-1}). Oroxylin A and diazepam were administered p.o. 1 hr and i.p. 15 min prior to picrotoxin, respectively. The following parameters were recorded: (i) number of mice with convulsions (%), (ii) latency to the onset of the first jerk or clonus after injection, (iii) death rate, and (iv) latency to death. A clonic seizure was defined as forelimb clonus of >3-s duration. Latencies represent the time, in minutes, between PTX injection and the observed parameters.

2.5.9. Data analysis

Behavioral data obtained from each response were subjected to one-way ANOVA, and multiple group comparisons were made by Dunnett's *t*-test for only those responses which yielded significant treatment effects in the ANOVA test. Fisher's exact test and Bonferroni's multiple comparison test were used when necessary. Significance was reported starting at the 0.05 level.

3. Results

3.1. Radioreceptor binding assays

Oroxylin A inhibited [^{3}H]flunitrazepam binding to the rat cerebral cortex membranes with a IC_{50} of 1.09 ± 0.07 μM

Table 1
Determination of IC_{50} and GABA ratio of oroxylin A by [^{3}H]flunitrazepam binding assay and GABA shift experiment

Drug	Inhibition of [^{3}H]flunitrazepam (μM) IC_{50}	GABA ratio
Oroxylin A	1.09 ± 0.07	1.09 ± 0.04
Diazepam	0.008 ± 0.0002	2.24 ± 0.24
Flumazenil	0.006 ± 0.0003	0.91 ± 0.01

IC_{50} values were estimated by displacement of [^{3}H]flunitrazepam binding to synaptosomal membrane protein extracted from cerebral cortex of Sprague–Dawley rat (approximately 250 g). GABA ratios are determined in presence of 10 μM GABA with extensively washed synaptosomal membrane preparation from rat cerebral cortex employing 1 nM [^{3}H]Ro15-1788. Data represent mean ± SEM, N = 3.

and manifested a GABA shift of 1.09 ± 0.04 (Table 1), suggesting that this flavonoid might interact as an antagonist at the BDS. Scatchard plot analysis of [^{3}H]flunitrazepam binding showed that oroxylin A at 1 and 5 μM decreased the binding affinity (K_d) with no change in the number of binding sites (B_{max}), indicative of competitive inhibition of [^{3}H]flunitrazepam binding at the BDS (Fig. 2). In order to examine the *in vivo* effects of oroxylin A, animal models routinely employed for BDS ligand evaluations were performed.

3.2. Behavioral studies

3.2.1. Locomotor activity test

Oroxylin A-treated mice did not exhibit any changes in the number of transitions across photocells in comparison to vehicle. Likewise, diazepam at 1 mg kg^{-1} did not result in significant changes in mice in this regard (Table 2).

3.2.2. Holeboard test

No significant difference was observed in the number of head-dips made and the number of rears in oroxylin A (3.75–60 mg kg^{-1}) and diazepam-treated (1 mg kg^{-1}) mice in comparison to control, suggesting that no sedative

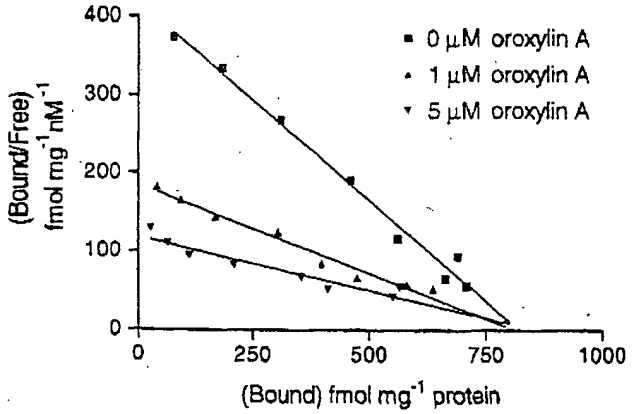

Fig. 2. A representative Scatchard plot of [^{3}H]flunitrazepam (0.1–10 nM) binding to rat cerebral cortex benzodiazepine receptor *in vitro* in the absence and presence of oroxylin A (1 and 5 μM). Data represents mean of three individual experiments done in triplicates.

Table 2
Assessment of locomotor activity of oroxylin A-treated mice

Drug (mg kg^{-1})	Locomotor activity counts
Vehicle	238.6 ± 25.6
DZ (1)	286.3 ± 24.6
Oroxylin A (3.75)	292.6 ± 29.3
Oroxylin A (7.5)	281.1 ± 25.4
Oroxylin A (15)	286.8 ± 31.2
Oroxylin A (30)	266.9 ± 16.9
Oroxylin A (60)	285.0 ± 21.3

Locomotor activity counts (mean ± SEM) in mice during a 5-min period 1 hr after oral administration of vehicle (Veh, ddH_2O), diazepam (DZ, 1 mg kg^{-1}) or oroxylin A (3.75–60 mg kg^{-1}), N = 8–12 mice per group.

Table 3
Exploratory behavior of mice treated with oroxylin A in the holeboard test

Drug (mg kg^{-1})	Number of head-dips	Number of rears
Vehicle	20.23 ± 3.36	26.00 ± 2.99
DZ (1)	22.78 ± 1.77	24.22 ± 1.15
Oroxylin A (3.75)	22.58 ± 3.62	22.08 ± 4.45
Oroxylin A (7.5)	24.44 ± 5.14	22.11 ± 4.21
Oroxylin A (15)	19.50 ± 3.18	20.63 ± 4.24
Oroxylin A (30)	12.33 ± 4.09	23.00 ± 2.28
Oroxylin A (60)	19.30 ± 3.27	26.00 ± 3.37

Number of head-dips and rears made in mice orally administered oroxylin A (3.75, 7.5, 15, 30, 60 mg kg^{-1}), diazepam (DZ, 1 mg kg^{-1}) or vehicle (water). Data represent mean ± SEM, N = 16.

effect was elicited at these dosages (Table 3). On the other hand, at 3 mg kg^{-1} i.p. diazepam, there were significantly decreased numbers of head-dips and rears. Pretreatment of oroxylin A (15–60 mg kg^{-1}) did not significantly affect the diazepam-induced sedation (3 mg kg^{-1}, i.p.) as measured by the number of head-dips and rears (Table 4).

3.2.3. Elevated plus-maze

Oroxylin A-treated mice (3.75–60 mg kg^{-1}) did not exhibit any significant differences with respect to the percentage of open arms entries, the percentage of time spent in open arms and the number of closed arm entries in comparison to control mice (Fig. 3a). In contrast, diazepam-treated mice (1 mg kg^{-1}) demonstrated significant

Table 4
Assessment of exploratory behavior of oroxylin A-treated mice in the holeboard test

Drug (mg kg^{-1})	Number of head-dips	Number of rears
Vehicle	17.94 ± 2.81	20.94 ± 3.78
DZ (3)	4.23 ± 1.33**	7.00 ±1.38**
DZ (3) + oroxylin A (15)	5.13 ± 1.25**	6.88 ± 3.17**
DZ (3) + oroxylin A (30)	10.00 ± 0.53*	5.08 ± 3.46**
DZ (3) + oroxylin A (60)	5.14 ± 2.28**	2.93 ± 1.16**

Data represent (mean ± SEM) the number of head-dips and rears made in mice pretreated with oroxylin A (0, 15, 30, 60 mg kg^{-1}, p.o.) 15 min prior to diazepam (3 mg kg^{-1}, i.p.), or vehicle (10% DMSO, i.p.). *$P < 0.05$, **$P < 0.01$, significantly different from control, Bonferroni's multiple comparison test after one-way ANOVA, N = 8–12 mice per group.

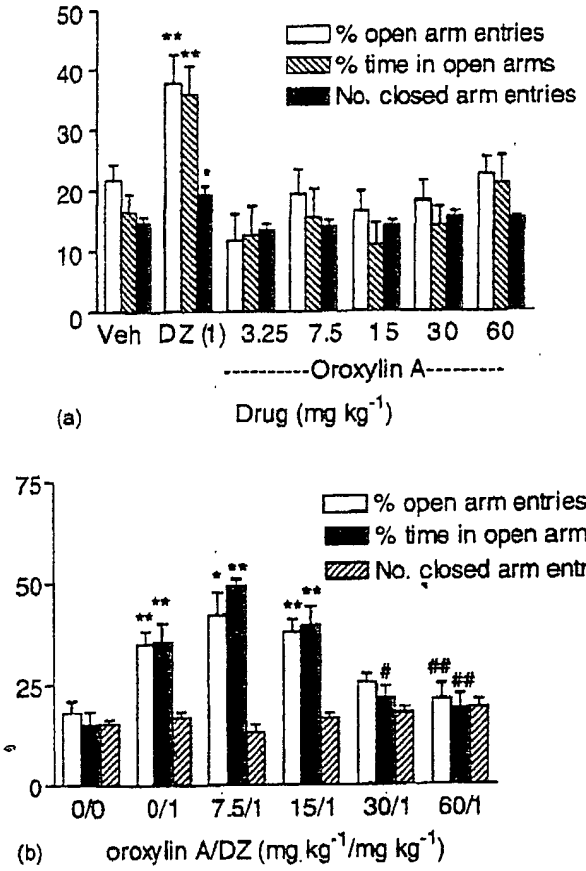

Fig. 3. (a) Assessment of the anxiolytic effect of oroxylin A in the mice elevated plus-maze. Data represent mean ± SEM the percentage of entries and time spent in open arms, and the number of closed arm entries in mice 1 hr after oral administration of oroxylin A (3.75, 7.5, 15, 30, 60 mg kg^{-1}), diazepam (DZ, 1 mg kg^{-1}) or vehicle (ddH_2O, pH 7.4). $^{*}P < 0.05$, $^{**}P < 0.001$ significantly different from control, Dunnett's t-test after one-way ANOVA, N = 8–12 mice per group. (b) Antagonistic effect of oroxylin A pretreatment in the mice elevated plus-maze. Data represent mean ± SEM the percentage of entries and time spent in open arms, and the number of closed arm entries in mice co-administered oroxylin A (0, 7.5, 15, 30, 60 mg kg^{-1}, p.o.) and diazepam (DZ, 1 mg kg^{-1}, i.p.), or vehicle (10% DMSO, i.p.), $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from Veh, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ significantly different from DZ, Bonferroni's multiple comparison test after one-way ANOVA, N = 8–12 mice per group.

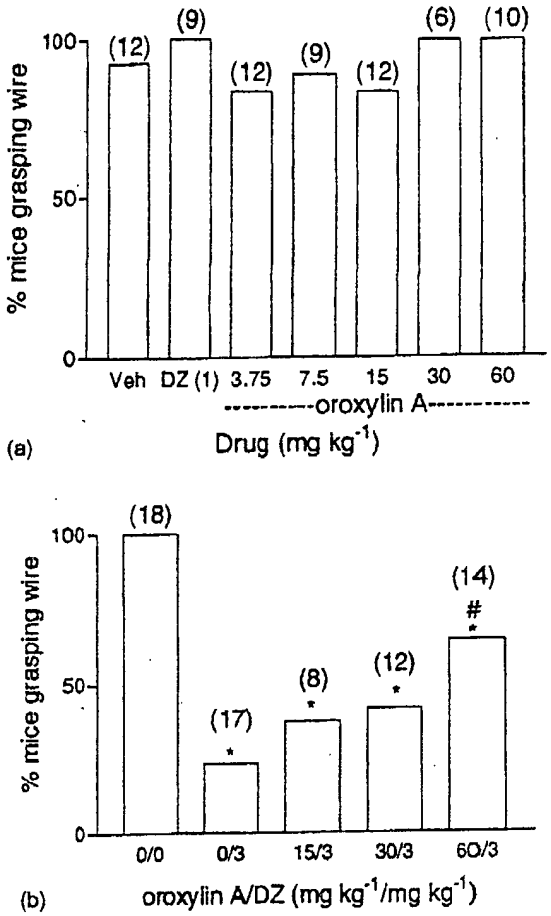

Fig. 4. (a) Performance of mice in the horizontal wire test. Percentage of mice grasping the wire after oral administration of oroxylin A (3.75, 7.5, 15, 30, 60 mg kg^{-1}), diazepam (DZ, 1 mg kg^{-1}) or vehicle (Veh, ddH_2O), number of mice per group in parentheses. (b) Reversal by oroxylin A pretreatment of the diazepam-induced myorelaxation in the horizontal wire test. Percentage of mice grasping the wire after co-administration of oroxylin A (0, 15, 30, 60 mg kg^{-1}, p.o.) and diazepam (DZ, 3 mg kg^{-1}, i.p.), or vehicle (10% DMSO, i.p.); number of mice per group in parentheses. $^{*}P < 0.01$, significantly different from Veh, $^{\#}P < 0.05$ significantly different from DZ, Fisher's exact test.

anxiolysis as observed in the selective increases in both open-arm parameters, with a slight increase in the total number of closed arm entries, thus indicating increased locomotor activity. Pretreatment of oroxylin A (30 and 60 mg kg^{-1}) reversed both diazepam-elicited (1 mg kg^{-1}, i.p.) open-arm parameters to basal levels, demonstrating that the anxiolytic effect of diazepam was abolished in the presence of oroxylin A. Such reversals were not observed at lower doses of oroxylin A (7.5 and 15 mg kg^{-1}; Fig. 3b).

3.2.4. Horizontal wire test

Both oroxylin A (3.75–60 mg kg^{-1}) and diazepam (1 mg kg^{-1}) did not compromise the grasping of wire by mice when compared to control, pointing to a lack of myorelaxation at these dosages (Fig. 4a). Diazepam at 3 mg kg^{-1}, i.p., in contrast, elicited significant myorelaxation in mice as signified by the decrease in the percentage of mice grasping the wire. This diazepam-induced myorelaxation was relieved by pretreatment of 60 mg kg^{-1} of oroxylin A (Fig. 4b).

3.2.5. Rotarod test

Both oroxylin A (3.75–60 mg kg^{-1}) and diazepam (1 mg kg^{-1}) did not compromise the ability of mice to stay on the rotarod, revealing the absence of any motor incoordination effect (Fig. 5a). At 3 mg kg^{-1} diazepam, the percentage of mice that stayed on the rotarod decreased significantly, but this decrease was reversed by pretreatment of oroxylin A (60 mg kg^{-1}; Fig. 5b).

3.2.6. PTX-induced seizure test

In the picrotoxin-induced seizure test, oroxylin A (3.75–60 mg kg^{-1}) did not alter the onset of the first seizure, death latency, the percentage of convulsant animals or the

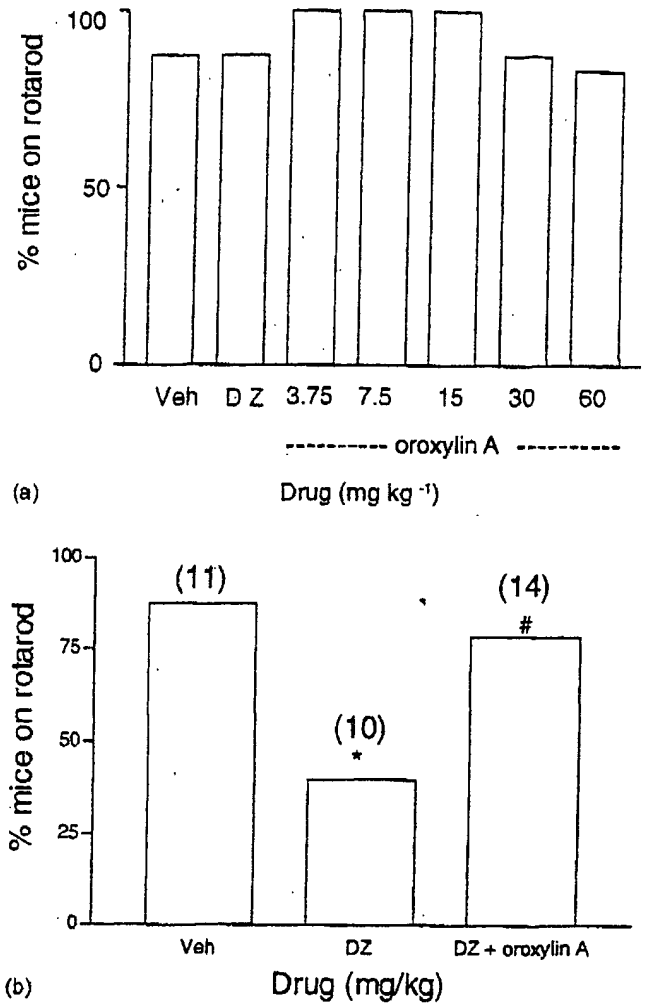

Fig. 5. (a) Performance of mice in the rotarod test. Percentage of mice that stayed on the rotarod 1 hr after oral administration of vehicle (Veh, ddH_2O), diazepam (DZ, 1 mg kg^{-1}) or oroxylin A (3.75–60 mg kg^{-1}), N = 8–12 mice per group. (b). Reversal by oroxylin A pretreatment of the diazepam-induced motor incoordination in the rotarod test. Percentage of mice that stayed on the rotarod after treatment of vehicle (10% DMSO, i.p.), diazepam (3 mg kg^{-1}, i.p.) alone or with oroxylin A (60 mg kg^{-1}, p.o.), $^*P < 0.01$ significantly different from Veh, $^\#P < 0.05$ significantly different from DZ, Fisher's exact test, number of mice per group in parentheses.

death rate. However, administration of 3 mg kg^{-1} diazepam significantly altered the latency of the first seizure in PTX-treated mice, the percentage of convulsant animals, and the death rate. When oroxylin A was administered prior to diazepam treatment (3 mg kg^{-1}), these parameters did not change significantly in comparison to the diazepam-treated mice (Table 5).

4. Discussion

Flavonoids, an important class of naturally occurring compounds found in many vascular plants, have demonstrated CNS activities, interacting with a number of receptor systems in the CNS [23–25]. Ligand binding at the BDS on the $GABA_A$ receptor complex are known to exert such pharmacological actions as anxiolysis, anticonvulsion, muscle relaxation and sedation [26]. Our search for BDS ligands from traditional herbal medicinal sources has led to the identification of several flavonoid derivatives from *S. baicalensis* Georgi with BDS activities [15]. In the present study, an additional flavonoid, oroxylin A, was found to be an orally active BDS ligand with some antagonistic properties.

In radioligand displacement studies employing [^{3}H]flunitrazepam, oroxylin A exhibited moderate affinity for the BDS, and in the saturation experiments where oroxylin A (1 and 5 μM) was incubated with different concentrations of [^{3}H]flunitrazepam, the ability of oroxylin A to modify the K_d without significant changes in the B_{max} suggests that this flavonoid derivative interacts competitively at the recognition site. A GABA ratio of 1.09 also suggests that oroxylin A behaves in a manner similar to that of a BDS antagonist (Table 1). To characterize the pharmacological profile of this flavonoid and to substantiate the extrapolation made utilizing these *in vitro* assays, a range of behavioral tests were conducted.

In the locomotor activity test, oroxylin A-treated mice did not exhibit any changes in comparison to control,

Table 5
Assessment of anticonvulsant properties of oroxylin A in the PTX-induced seizure model

Drug (mg kg^{-1})	Latency to first tonic-clonus seizure (min)	Death latency (min)	% Convulsant animals	Death rate
Vehicle	9.4 ± 1.1	16.1 ± 1.6	100	90
DZ (3)	22.8 ± 1.0*	–	18*	0*
Oroxylin A (3.75)	7.2 ± 0.9	17.8 ± 2.0	100	89
Oroxylin A (7.5)	8.7 ± 0.6	18.0 ± 1.6	100	83
Oroxylin A (15)	9.3 ± 0.5	19.1 ± 1.7	100	100
Oroxylin A (30)	11.1 ± 1.0	17.8 ± 1.2	100	100
Oroxylin A (60)	11.1 ± 1.4	18.6 ± 1.4	100	88
DZ (3) + oroxylin A (60)	22.9 ± 0.9*	–	25*	0*

Values represent (mean ± SEM) latency to the first tonic-clonic seizure, death latency, percentage of convulsant animals and death rate in mice after administration of vehicle, diazepam (DZ, 3 mg kg^{-1}, i.p.), oroxylin A (3.75–60 mg kg^{-1}, p.o.) or diazepam (DZ, 3 mg kg^{-1}, i.p.) and oroxylin A (60 mg kg^{-1}, p.o.). Diazepam and oroxylin A are administered 15 min and 1 hr prior to picrotoxin injections, respectively, $^*P < 0.001$ significantly different from control, Dunnett's *t*-test after one-way ANOVA or Fisher's exact test, N = 8–12 per group.

indicating that oroxylin A has no stimulant or depressant effects at the test doses (Table 2). In the holeboard test, an assay for potential sedative effects, oroxylin A, likewise, did not induce any change in the numbers of head-dips and rears made (Table 3). This flavonoid also did not give rise to any significant changes in the anxiolytic parameters as measured in the elevated plus-maze or any myorelaxant effects as observed in the horizontal wire test (Figs. 3a and 4a). Moreover, oroxylin A alone did not result in any motor incoordination as assessed in the rotarod test (Fig. 5a) and did not modify the parameters measured in the PTX-induced seizure test (Table 5). These results were entirely consistent with those expected of BDS antagonists.

To test if oroxylin A is capable of antagonizing the effects of the BDS agonist diazepam, it was administered to mice prior to the injection of two different dose levels of diazepam. Diazepam at 1 mg kg^{-1} brought about evident anxiolytic effect as seen in the increases in the percentage of number of open-arm entries and time spent in open arms. However, these effects were abolished by oroxylin A, thereby revealing the antagonistic effect of this flavonoid *via* the BDS (Fig. 3b). Mice treated with 3 mg kg^{-1} diazepam manifested sedative and myorelaxant effects in the holeboard test and the horizontal wire test, respectively. In contrast to abolishing the diazepam-induced anxiolysis, pretreatment of oroxylin A failed to block the sedation but partially reversed the myorelaxation caused by BDS agonist (Table 4 and Fig. 4b). The diazepam-elicited motor incoordination on the rotarod was also counteracted by 60 mg kg^{-1} oroxylin A (Fig. 5b). In the PTX-induced seizure test, 3 mg kg^{-1} diazepam demonstrated anticonvulsant action as measured in significant differences in the onset of the first seizure, the percentage of convulsant animals, death latency and death rate when compared to control. Oroxylin A (3.75–60 mg kg^{-1}), when administered alone, did not manifest any anticonvulsant properties. Likewise, oroxylin A pretreatment also failed to modify the diazepam-induced anticonvulsion in the chosen dosage regimen (Table 5).

In this study, the GABA shift assay, a useful means to characterize the pharmacological action of BDS ligands, was employed to characterize that of oroxylin A. Oroxylin A exhibited negligible changes in its binding affinity for the BDS in the presence or absence of GABA, a behavior resembling to that of an antagonist, which is in contrast to the expected enhancement by GABA of the binding affinity of a BDS agonist and reduction of that of an inverse agonist. Recent genetic knock-out and knock-in studies in mice, suggest that the sedative, amnesic and part of the anticonvulsant effects of $GABA_A$ receptor ligands may be mediated by the α_1 subunit, and the anxiolytic, myorelaxant, motor impairment and ethanol potentiation actions mediated by the $\alpha_{2,3,5}$ subunits [27–31]. The pharmacological profile of oroxylin A as observed in the present study, with opposition to the diazepam-induced anxiolytic, myorelaxant and motor incoordination effects, but not the sedative and anticonvulsant effects elicited by this BDS agonist, suggests that oroxylin A manifested antagonistic action mediated by the $\alpha_{2,3,5}$-containing BDS. Further studies on the binding affinity of oroxylin A for different $GABA_A$ receptor subunits will be needed to clarify its exact subtype selectivity.

In summary, our study demonstrated oroxylin A as the first known instance of a naturally occurring flavonoid possessing antagonistic properties at the BDS of the $GABA_A$ receptors. The study also highlights the pharmacological selectivity of oroxylin A for antagonizing various physiological actions induced by diazepam. Together with earlier findings on both the selectivity and the wide spectrum of efficacies amid the class of compounds for the BDS-mediated pharmacology, our observation is consistent with the notion that flavonoids are capable of exerting a broad spectrum of intrinsic activities at the recognition site, and represents a promising class of compound for the treatment of BDS-associated syndromes. Since the initial search for alternative BDS ligands amongst the flavonoids, a list of successful semi-synthetic analogues has been generated with enhanced affinities for the BDS by the incorporation of different side chains onto the flavone backbone [4,32]. Molecular modeling of flavonoid binding to the BDS pharmacophore has also been carried out in attempts to further increase the affinity of these derivatives [33–35], however, much is awaited for the investigation of subunit selectivity manifested by these compounds. The structure–function relationships of flavonoids deserve to be thoroughly and systematically investigated.

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